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PRINCIPAL INVESTIGATOR: Yin-Yuan Mo, Ph.D.

CONTRACTING ORGANIZATION: Southern Illinois University
Springfield, Illinois 62794-9616

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Yin-Yuan Mo, Ph.D.

E-Mail: ymo@siumed.edu

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14. ABSTRACT

The newly discovered RNA interference is a novel type of gene regulation mechanism, which is required for normal expression of genes. This study tests the hypothesis that breast tumor carries dysregulated RNA interference pathways, and thus, some tumor suppressor genes will be down-regulated while other genes (e.g., oncogenes) will be up-regulated, leading to tumor cell proliferation and survival. Using real time RT-PCR, we demonstrate that microRNA-21 is overexpressed in breast tumors compared to the matched normal breast tissue. Furthermore, we show that antisense oligonucleotide against microRNA-21 can suppress the endogenous microRNA-21 and causes tumor cell growth inhibition. Experiments with a xenograft carcinoma mouse model reveal that the antisense microRNA-21 oligonucleotide also inhibits tumor growth. Therefore, microRNA-21 is a potential therapeutic target for breast cancer therapy.

15. SUBJECT TERMS
RNA interference, RNAi, microRNA, breast tumorigenesis

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Introduction

Breast cancer, like other types of cancers, is frequently caused by altered gene expressions. The newly discovered RNA interference is a novel type of gene regulation mechanism. There are two types of small RNAs that are processed by the RNA interference pathway, short interfering RNAs (siRNAs) and microRNAs (miRNAs), both of which can silence gene expression at the post-transcriptional level. As a new layer of gene regulation mechanism, we believe that dysregulation of RNA interference pathway can cause alterations of gene expression in such a way that it contributes to breast cancer progression. The purpose of this study is to determine whether breast cancer cells reveal aberrant expression of such small RNA molecules.

Body

miR-21 is overexpressed in breast tumor tissues compared to the matched normal breast tissues

Previous studies have shown that several miRNAs are aberrantly expressed in various types of cancers by miRNA array or northern blot (1-5). In this study, we profiled miRNA expression in matched normal breast and breast tumor tissues by TaqMan Real-time PCR using a newly released miRNA array from ABI (Forest City, CA). The array carries specific primer sets that allow for detection of 157 mature human miRNAs. This method uses stem–loop RT primers derived from mature miRNAs, followed by TaqMan PCR analysis; it is specific for detection of mature miRNAs (6, 7). Furthermore, this method can discriminate among related miRNAs that differ by as little as one nucleotide (6) and it is very sensitive such that it is possible to analyze miRNA expression in a single cell (8). Using the *miR-16* detection kit (for single miRNA) to optimize detection condition, we were able to detect *miR-16* from a range of several orders of input RNA and minimal amount of total RNA was at pg level (not shown), consistent with the previous report (6). We used U6 RNA for normalization of expression in different

samples. From a total of five pairs of matched advanced breast tumor tissue specimens, *miR-21* was the most abundantly expressed miRNA among all miRNAs in this array and moreover, the level of *miR-21* was much higher in the tumor tissues than in the matched normal tissues (Fig. 1A). Since one C_T (Threshold Cycle) unit is equivalent to ~2 fold difference (6), this conversion would result in over a 5-fold increases in *miR-21* levels for tumor tissues compared to the matched normal tissues after normalization to U6 RNA (Fig. 1B). Furthermore, using the individual *miR-21* primer set, we were able to confirm these results in more matched breast tumor samples (not shown).

Anti-miR-21 inhibits cell growth *in vitro*

Overexpression of *miR-21* in the breast tumor tissues suggest that *miR-21* may function as an oncogene. To test this possibility, we examined the effect of suppression of *miR-21* on breast tumor cell growth. Thus, we used anti-miR-21 inhibitor since this approach has been successfully used to inhibit *miR-21* (9, 10). The anti-miR-21 inhibitor is a sequence-specific and chemically modified oligonucleotide to specifically target and knockdown *miR-21* molecule. Both anti-miR-21 and negative control (scrambled oligonucleotides) were obtained from Ambion (Austin, TX). TaqMan Real-time PCR revealed that anti-miR-21 significantly reduced *miR-21* level (Fig. 2A), suggesting that anti-miR-21 is efficiently introduced into the cells and knock down *miR-21*. This is probably due to the formation of highly stable complexes of *miR-21* with anti-miR-21 that prevents miRNA detection by TaqMan Real-time PCR. Of interest, we found that anti-miR-21 reduced cell growth in a dose dependent manner. At 50 nM, the growth inhibition by anti-miR-21 reached about 25%, at day 3 after transfection (Fig.2B); this result was also in agreement with the previous report that *miR-21* inhibitors decrease human glioblastoma cell survival

(10). To further assess the effect of anti-miR-21 on cell growth, we treated the transfected cells with the anticancer drug topotecan (TPT) that is known to inhibit DNA topoisomerase I and cause DNA damage (11). Anti-miR-21-mediated cell growth inhibition was increased up to 40% when the transfected cells were treated with 0.1 μM TPT (Fig.2C). Therefore, anti-miR-21 can inhibit cell growth *in vitro.* These results also suggest that suppression of *miR-21* can sensitize tumor cells to anticancer agents.

Anti-miR-21 inhibits tumor growth in the xenograft carcinoma mouse model

Although it has previously been shown that there is a direct correlation between aberrant expression of *miR-21* and human malignancy (10, 12), it is not clear whether suppression of *miR-21* alone will affect tumorigenesis. Therefore, we transiently transfected MCF-7 cells with anti-miR-21 or the negative control, and then injected them into mammary pads of female nude mice. Of considerable interest, we found that tumors derived from MCF-7 cells transfected with anti-miR-21 grew substantially slowly, compared to the negative control during the whole tumor growth period (Fig.3A). By day 28 when tumors were harvested, average weight for tumors derived from cells transfected with anti-miR-21 was only about half of those derived from the cells transfected with the negative control (Fig.3B). Immunostaining with the anti-Ki-67 indicated that the reduced tumor growth is likely due to a lower proliferation caused by anti-miR-21 because Ki-67 staining was much weaker for anti-miR-21 than for the negative control (Fig.3C). These results strongly suggest that *miR-21* plays an important role in tumorigenesis. Of interest, the inhibitory effect of anti-miR-21on tumor growth (Fig.3B) is greater (about 50%), compared to its inhibitory effect on cell growth *in vitro* (about 25%) (Fig.2A). Although the observation time for tumor growth is longer than *in vitro* cell growth inhibition

assays, which could explain in part the difference, other factors could also contribute to this difference. For instance, stress from the tumor microenvironment, such as hypoxia, may enhance the inhibitory effect of the anti-miR-21. This appears to be in agreement with the finding that other stresses, such as DNA damage caused by TPT, can increase the inhibitory effect mediated by anti-miR-21 (Fig.2B). Alternatively, anti-miR-21 could also affect genes that are linked to other tumorigenesis factors, which might explain in part why more inhibition for anti-miR-21 was seen in tumors than cell growth *in vitro*.

Anti-miR-21 increases cell apoptosis and downregulates *bcl-2* expression

To dissect the molecular basis underlying this *miR-21*-associated alteration of tumor growth, we searched for potential *miR-21* targets using programs available (e.g., http://microrna.sanger.ac.uk/targets/v2/; http://genes.mit.edu/cgi-bin/targetscan_lookup2.pl?KEYWORD=miR-21) and tested several genes that are likely involved in tumorigenesis such as FasL. However, their protein levels were not affected by anti-miR-21 (not shown). Thus, we tested whether anti-miR-21 suppresses cell growth by triggering apoptosis pathways since previous studies have suggested that *miR-21* regulates apoptosis pathways in tumor cells (10). Consistent with the previous report for glioblastoma cells (10), but contrary to the results in HeLa cells (9), we found that anti-miR-21 caused more apoptosis than the negative control in MCF-7 cells by a 4.5-fold (Fig.4A). To further determine the possible involvement of apoptosis in anti-miR-21-mediated growth inhibition we treated transfected cells with the general caspase inhibitor Z-VAD-fmk. As shown in Fig. 4B, Z-VAD-fmk was able to reverse the growth inhibition caused by anti-miR-21, suggesting that increased apoptosis in the anti-miR-21-treated MCF-7 cells is at least in part responsible for the observed growth inhibition.

Furthermore, we detected a lower level of Bcl-2 protein in the anti-miR-21-transfected MCF-7 cells (Fig.4C, left panel) as well as tumors derived from the MCF-7 cells transfected with anti-miR-21 (Fig.4C, right panel). Thus, the induction of apoptosis by anti-miR-21 is possibly in part due to downregulation of Bcl-2. We also examined *bcl-2* mRNA by RT-PCR and found that *bcl-2* mRNA was decreased in the anti-miR-21-treated cells (Fig.4D), suggesting that *miR-21* may regulate *bcl-2* expression indirectly. Although we cannot exclude the possibility that anti-miR-21 may cause degradation of *bcl-2* mRNA, based on our current knowledge of miRNA-mediated gene regulation (13, 14), one possibility would be that anti-miR-21 targets and suppresses expression of a gene(s) that negatively regulates *bcl-2* expression. Reduction of a protein encoded by such a *miR-21*-targeting gene(s) would cause upregulation of both *bcl-2* mRNA and protein whereas anti-miR-21 would have an opposite effect on both *bcl-2* mRNA and protein. Therefore, identification of direct *miR-21* targets may provide new insight into how *miR-21* controls expression of genes, such as *bcl-2*.

Key Research Accomplishments

- We profiled miRNA expression in matched normal breast tissue and breast tumor tissues by TaqMan Real-time PCR miRNA array methods.
- We found that *miR-21* was highly overexpressed in breast tumors compared to the matched normal breast tissues among 157 human miRNAs analyzed.
- Anti-miR-21 suppressed both cell growth *in vitro* and tumor growth in the xenograft mouse model.
- This anti-miR-21-mediated cell growth inhibition was associated with increased apoptosis and decreased cell proliferation, which could be in part due to downregulation of the anti-apoptotic Bcl-2 in anti-miR-21-treated tumor cells.

- Together, these results suggest that *miR-21* functions as an oncogene and modulates tumorigenesis through regulation of genes such as *bcl-2* and thus, it may serve as a novel therapeutic target.

Reportable Outcomes

Abstract presented in 2006 AACR meeting entitled "Suppression of tumor growth by anti-miRNA21" April, 2006, Washington DC

Conclusions

We have demonstrated that miRNA-21 is overexpressed in breast tumor compared to the matched normal breast tissues. More importantly, suppression of miR-21 by antisense oligonucleotide inhibits breast tumor growth. Therefore, the antisense miR-21 oligonucleotdie may prove a potent therapeutic agent. Future work will be to understand molecular mechanism by which miR-21 impacts on breast cancer.

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Figure legend

Fig.1 Expression of *miR-21* in matched normal and breast tumor tissues. Total RNA was extracted by the Trizol reagent (Invitrogen) and miRNAs were detected by the TaqMan Real-time PCR microRNA array. Freshly frozen human nonneoplastic normal breast tissue and matched breast tumor samples were obtained from the SIU Cancer Institute Tumor Bank and National Cancer Institute-sponsored Cooperative Human Tissue Network (CHTN, http://www-chtn.ims.nci.nih.gov/), respectively. TaqMan microRNA assays used Human Panel-Early Access Kit (ABI, Forest City, CA) which includes 157 human miRNAs as well as 3 negative controls. For RT reactions, 10

ng total RNA was used in each reaction (15 μl) and mixed with corresponding TaqMan microRNA assays RT primer (3 μl). The RT reaction was performed at the following conditions: 16°C for 30 min; 42°C for 30 min; 85°C for 5 min, and then hold on 4°C. After the RT reaction, the cDNA products were diluted at 15, 150 and 1,500 x, respectively, and 1.33 μl diluted cDNA was used for PCR reaction along with TaqMan primer (2μl). The PCR reaction was carried out at 95°C for10 min, followed by 40 cycle of 95°C for 15 sec and 60°C for 60 sec. To detect suppression of *miR-21* expression by anti-miR-21, the following primers were used (Chen et al., 2005): RT primer GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA; PCR forward primer GCCCGCTAGCTTATCAGACTGATG; PCR reverse primer GTGCAGGGTCCGAGGT and TaqMan probe (6-FAM)- CTGGATACGACTCAACA-(MGB). Primers for detection of U6 RNA expression were U6-5, GTGCTCGCTTCGGCAGCACATAT and U6-3.1, AAAAATATGGAACGC TTCACGAA. Relative expression was expressed as C_T (**A**) or fold change after normalization to U6 RNA (**B**). Values are means of 5 pairs of matched breast tumor samples ±SE. **, $p<0.01$.

Fig.2 Inhibition of cell growth by anti-miR-21 oligonucleotides. A, Suppression of *miR-21* expression by anti-miR-21 as detected by TaqMan Real-time PCR. MCF-7 cells were transfected with the negative control (Ambion) or anti-miR-21 (Ambion) at 50 μM using Optifect Reagent (Invitrogen) following the manufacture's protocol. Total RNA was isolated from the transfected cells 3 days after transfection, followed by TaqMan Real-time PCR assay. **B**, Cell growth inhibition. MCF-7 cells were transiently transfected

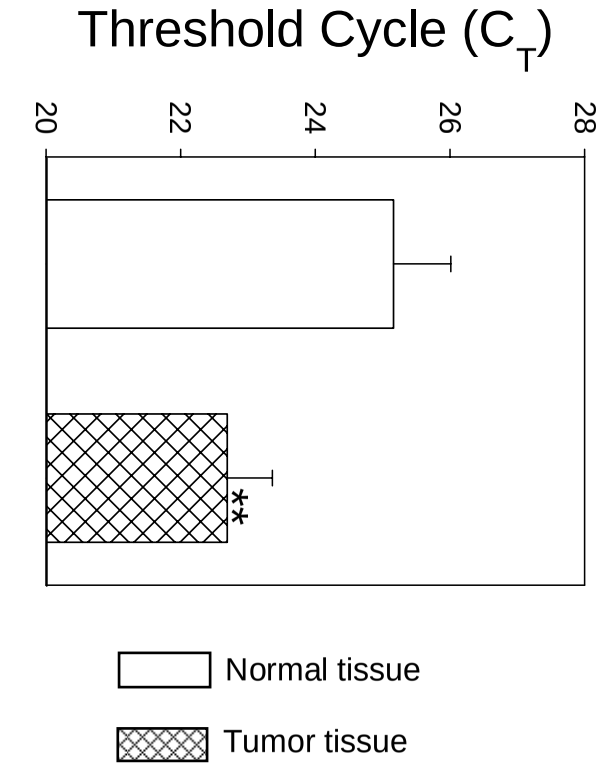
with the negative control or anti-miR-21 oligonucleotides at 50 nM and then were seeded in 96 well at 2500 cell/well. The cells were allowed to grow for 3 days before MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide) assay, as previously described (Mo et al., 2004). **C**, Cell growth inhibition in the presence of the anticancer agent topotecan (TPT). Cells were first transfected with 50 nM of negative control or anti-miR-21 and then treated with 0.1 μM of TPT for 3 days. Values in both **B** and **C** are means of three separated experiments ±SE. **, $p<0.01$. N, negative control; A, anti-miR-21.

Fig.3 Suppression of tumor growth by anti-miR-21 oligonucleotides. Female nude (nu/nu) mice (4-5 weeks old) were purchased from Harlan (Indianapolis, IN) and were maintained in the SIU School of Medicine's accredited animal facility. All animal studies were conducted in accordance with NIH animal use guidelines and a protocol approved by the SIU Animal Care Committee. MCF-7 cells were transfected with negative control (50 nM) or anti-miR-21 (50 nM) and then were harvested 2 days after transfection. The cells were mixed with 50% matrigel (BD Biosciences, San Jose, CA) at 15 million cells/ml and injected (1.5 million cells per spot) into mammary pads of female nude mice (two spots per mouse). To facilitate tumor growth, a 0.72mg 17β-estradiol pellet (Innovative Research of America, Sarasota, FL, USA) was implanted beneath the back skin. **A**, Tumor growth curves measured after injection of MCF-7 cells transfected with either the negative control or anti-miR-21 oligonucleotides. The tumor volume was calculated using the formula volume = $D \times d^2 \times \pi/6$ (Zhang et al., 2002), where D is the longer diameter, d is the shorter diameter. **B**, Representative tumor bearing mice 28 days

after inoculation. **C**, Tumor weight. Values in **A** and **C** are means of tumor volume or weight ±SE (negative control, n=14; anti-miR-21, n=16). **, $p<0.01$. **D**, Tumors derived from anti-miR-21-transfected cells revealed a lower level of Ki-67 antigen than the negative control. Frozen tumor samples were sectioned at a thickness of 6 μm and were stained with H&E staining or anti-Ki-67 (Invitrogen) according to standard methods.

Fig.4 Anti-miR-21-induced apoptosis and downregulation of Bcl-2. A, Detection of apoptosis in MCF-7 cells transfected with anti-miR-21 compared to the negative control. Apoptotic cell death was detected using cell death detection ELISA^{plus} kit (F. Hoffmann-La Roche Ltd, Basel, Switzerland) following manufacture's protocol. This detection kit is designed for detection of internucleosomal DNA fragmentation by antibody-mediated capture and cytoplasmic mononucleosome- and oligonucleosome-associated histone-DNA complexes (Liu et al., 2003). Signals were measured at 405 nm against ABTS solution as a blank and the relative apoptosis levels were calculated accordingly. **B**, Suppression of anti-miR-21-induced growth inhibition by Z-VAD-fmk. MCF-7 cells were transfected with the negative control or anti-miR-21 as in Fig.2A and then the inhibitor was added to the transfected cells one day after transfection. Three days later, cell growth inhibition was determined. **C**, Expression of Bcl-2 protein in anti-miR-21 in MCF-7 cells and tumors derived from MCF-7 cells transfected with the negative control or anti-miR-21, as detected by Western blot. N-1 and N-2 are tumors 1 and 2 derived from the negative control-treated MCF-7 cells, respectively; A-1 and A-2 are tumors 1 and 2 derived from the anti-miR-21-treated MCF-7 cells, respectively. **D**. Downregulation of *bcl-2* mRNA in the anti-miR-21-treated MCF-7 cells, as detected by

RT-PCR. PCR reaction was carried out using Bcl-2 primer set (Bcl-2-5.1 sense, ACCTGGATCCAGGATAACGGAG; Bcl-2-3.1 antisense, CCAACAACATGGAAAGCGAATC) at the following conditions: 94°C for 3 min, followed by 30 cycles of 94°C for 0.5 min, 54°C for 1 min and 72°C for 0.5 min. **C** and **D** are representatives of at least three experiments. Values in both **A** and **B** are means of three separate experiments ±SE. **, $p<0.01$. NS, not significant; N, negative control; A, anti-miR-21.


Threshold Cycle (C_T)
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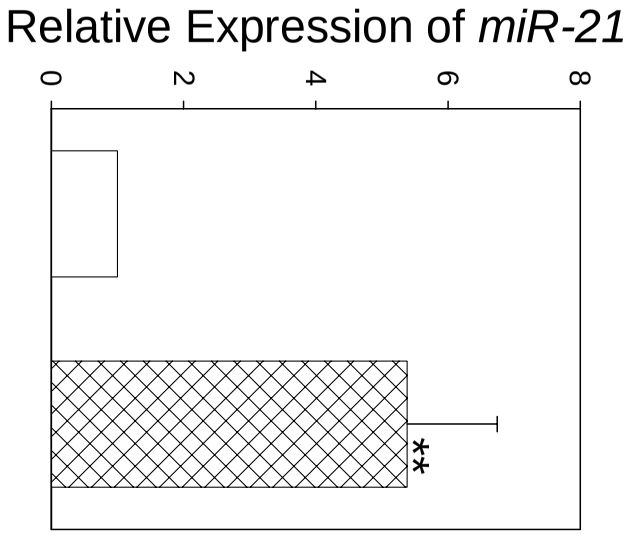
22
24
26
28
**
Normal tissue
Tumor tissue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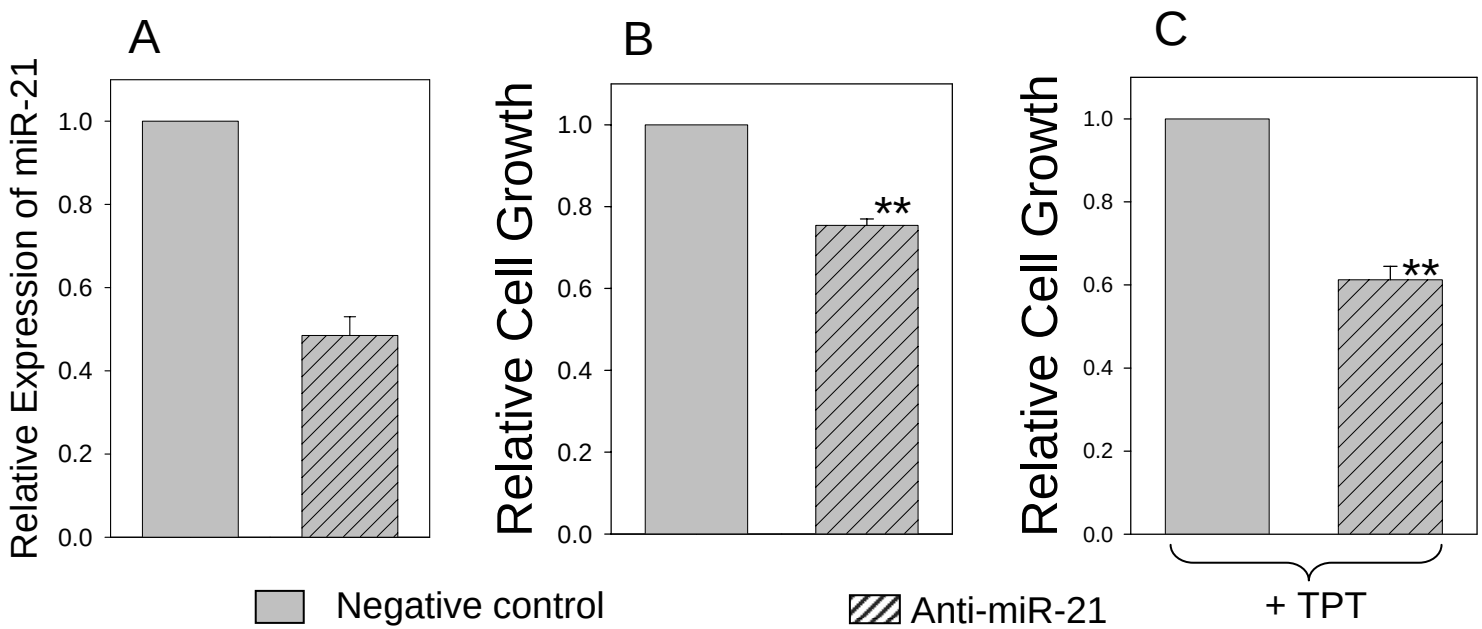
2
4
6
8
**


A
B
C
Relative Expression of miR-21
Relative Cell Growth
Relative Cell Growth
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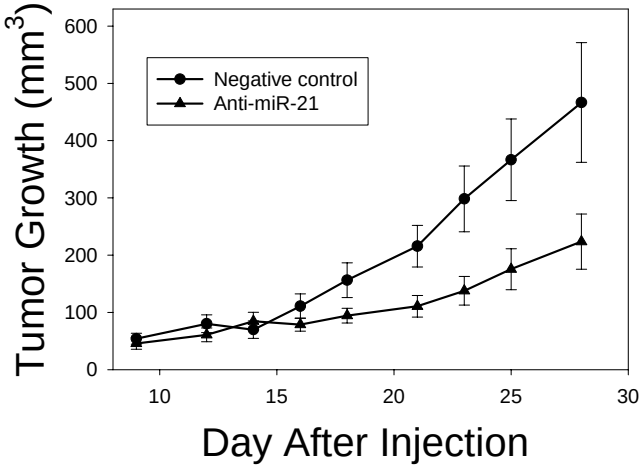
0.4
0.2
0.0
**
**
Negative control
Anti-miR-21
+ TPT

Fig.2

A


Tumor Growth (mm3)
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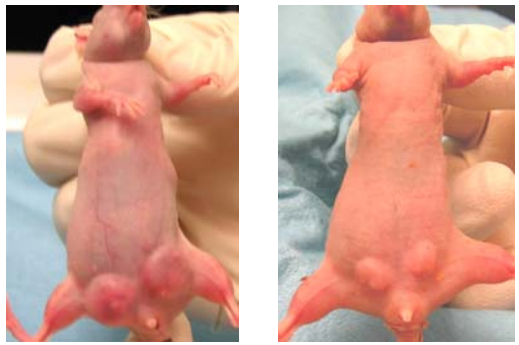
500
400
300
200
100
0
Negative control
Anti-miR-21
10
15
20
25
30
Day After Injection

B


Negative control
Anti-miR-21

C

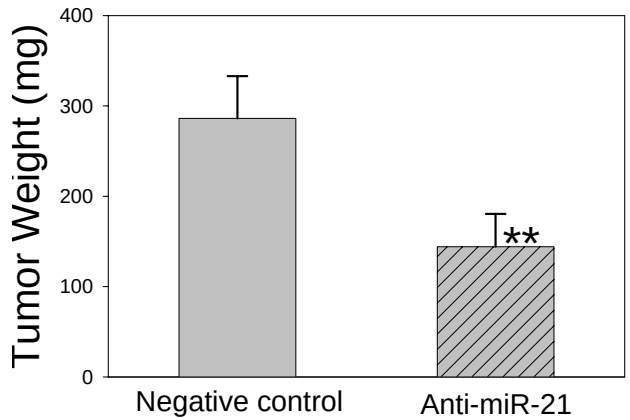

Tumor Weight (mg)
400
300
200
100
0
**
Negative control
Anti-miR-21

Fig.3

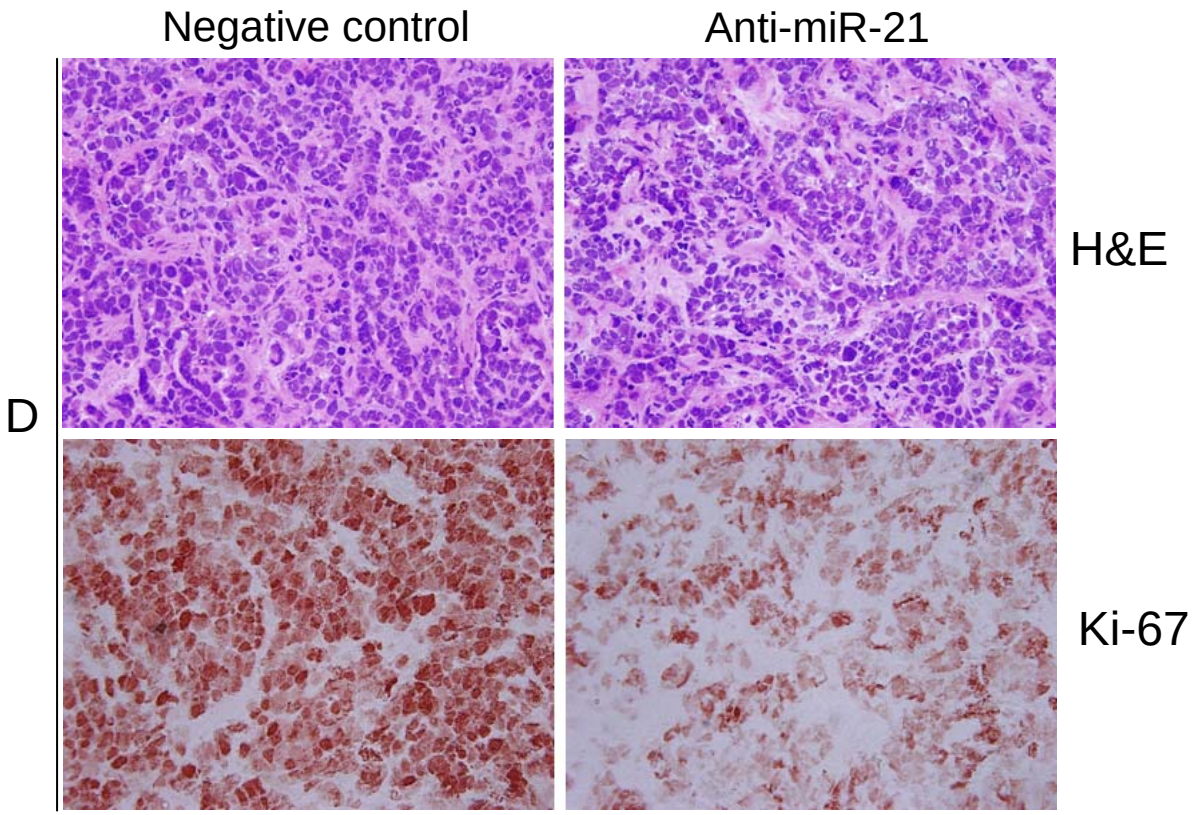
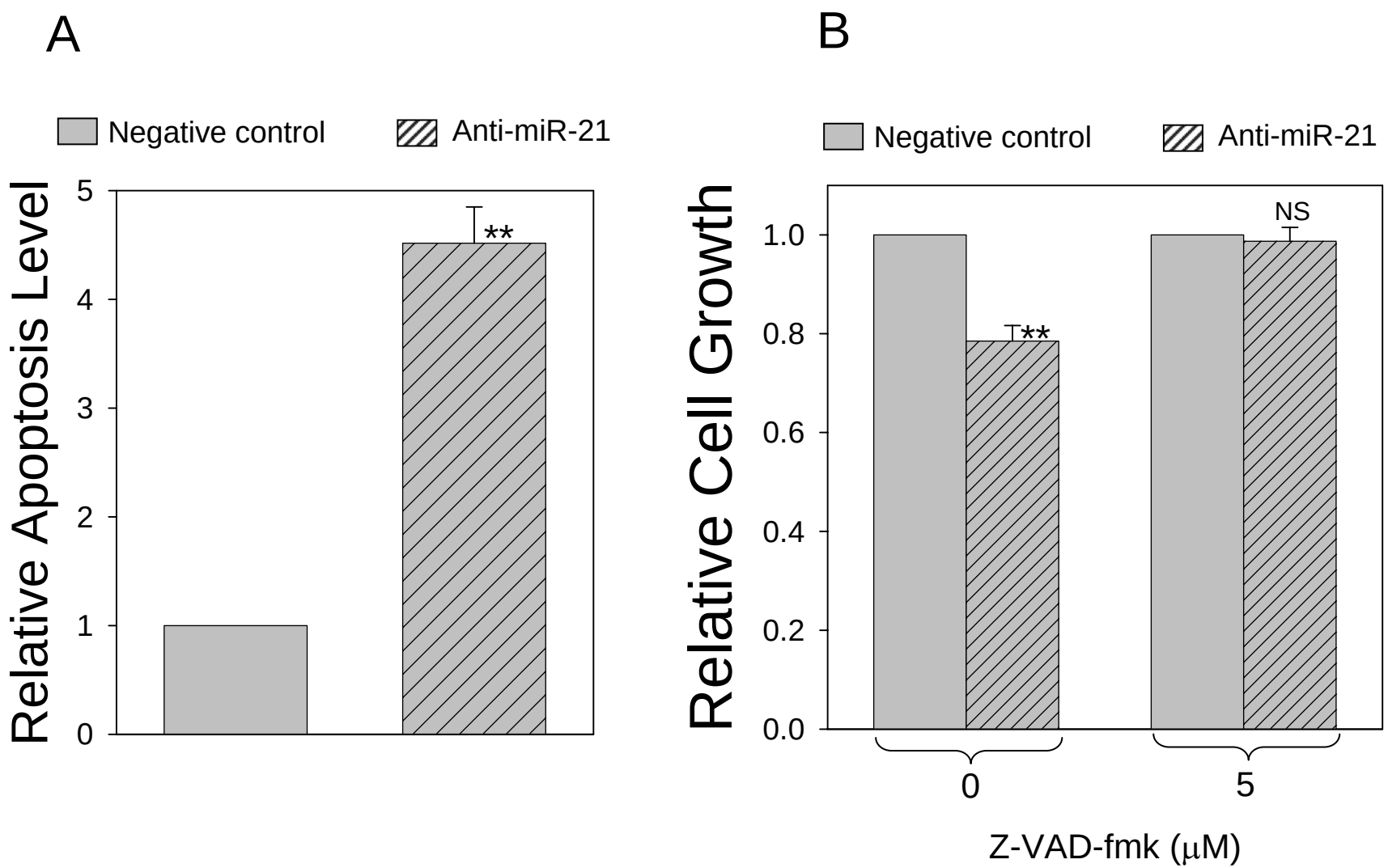


Fig.3


A
Negative control
Anti-miR-21
Relative Apoptosis Level
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1
2
3
4
5
**
B
Negative control
Anti-miR-21
Relative Cell Growth
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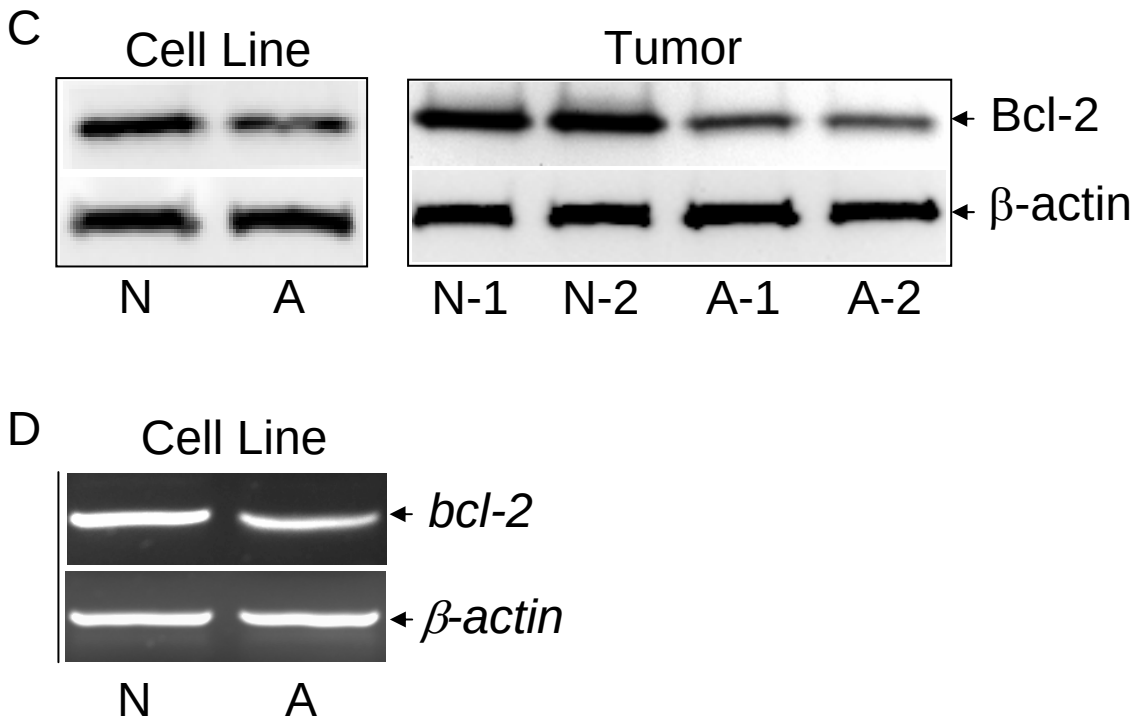
0.4
0.6
0.8
1.0
**
NS
0
5
Z-VAD-fmk (μM)

Fig.4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Cell Line
Tumor
Bcl-2
β-actin
N
A
N-1
N-2
A-1
A-2
D
Cell Line
bcl-2
β-actin
N
A

Fig.4